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Genetic and morphological insights into the *Carpobrotus* hybrid complex around the world

Ana Novoa[1], Heidi Hirsch[2], María L. Castillo[1], Susan Canavan[1,3], Luís González[4], David M. Richardson[1,5], Petr Pyšek[1,6], Jonatan Rodríguez[1,7], Lurdes Borges Silva[8,9,10], Giuseppe Brundu[11], Carla M. D'Antonio[12], Jorge L. Gutiérrez[13,14], Megan Mathese[15], Sam Levin[16,17], Luís Silva[8,9,10,18], Johannes J. Le Roux[5,19]

1 *Department of Invasion Ecology, Institute of Botany, Czech Academy of Sciences, 25243 Průhonice, Czech Republic* **2** *Lynedoch Road, Stellenbosch 7600 South Africa* **3** *School of Natural Sciences, Ollscoil na Gaillimhe, University of Galway, Galway, Ireland* **4** *Plant Ecophysiology Group: Invasive plants, Department of Plant Biology and Soil Sciences, Universidade de Vigo, 36310, Vigo, Spain* **5** *Centre for Invasion Biology, Department of Botany and Zoology, Stellenbosch University, Stellenbosch, South Africa* **6** *Department of Ecology, Faculty of Science, Charles University, Viničná 7, 12844 Prague, Czech Republic* **7** *CRETUS, Department of Functional Biology, Faculty of Biology, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain* **8** *CIBIO, Research Center in Biodiversity and Genetic Resources, InBIO Associate Laboratory, Pólo dos Açores, Universidade dos Açores, Campus de Ponta Delgada, Rua da Mãe de Deus, 9500-321 Ponta Delgada, Açores, Portugal* **9** *BIOPOLIS Program in Genomics, Biodiversity and Land Planning, CIBIO, Campus de Vairão, 4485-661 Vairão, Portugal* **10** *UNESCO Chair – Land Within Sea: Biodiversity & Sustainability in Atlantic Islands, Universidade dos Açores, Campus de Ponta Delgada, Rua da Mãe de Deus, 9500-321 Ponta Delgada, Açores, Portugal* **11** *Department of Agricultural Sciences, University of Sassari, 07100 Sassari, Italy* **12** *Environmental Studies Program, University of California, Santa Barbara 93106, USA* **13** *Facultad de Ciencias Exactas y Naturales, Instituto de Geología de Costas y del Cuaternario (IGCyC, UNMdP-CIC) and Instituto de Investigaciones Marinas y Costeras (IIMyC, UNMdP-CONICET), Universidad Nacional de Mar del Plata, Mar del Plata, Argentina* **14** *Grupo de Investigación y Educación en Temas Ambientales (GrIETA), Estación Biológica Las Brusquitas, San Eduardo del Mar, Argentina* **15** *Department of Botany & Zoology, Stellenbosch University, Matieland 7602, South Africa* **16** *Martin Luther University Halle-Wittenberg, Institute of Geobotany, Am Kirchtor 1, 06108 Halle (Saale), Germany* **17** *German Centre for Integrative Biodiversity (iDiv) Halle-Jena-Leipzig, Deutscher Platz 5e, 04103 Leipzig, Germany* **18** *Faculty of Sciences and Technology, University of the Azores, Rua da Mãe de Deus, 9500-321 Ponta Delgada, Portugal* **19** *School of Natural Sciences, Macquarie University, Sydney, NSW 2109, Australia*

Corresponding author: Ana Novoa (novoa.perez.ana@gmail.com)

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Abstract

The genus *Carpobrotus* N.E.Br. comprises between 12 and 25 species, most of which are native to South Africa. Some *Carpobrotus* species are considered among the most damaging invasive species in coastal dune systems worldwide. In their introduced areas, these species represent a serious threat to native species and significantly impact soil conditions and geochemical processes. Despite being well studied, the taxonomy of *Carpobrotus* remains problematic, as the genus comprises a complex of species that hybridize easily and are difficult to distinguish from each other. To explore the population genetic structure of invasive *Carpobrotus* species (i.e., *C. acinaciformis* and *C. edulis*) across a significant part of their native and non-native ranges, we sampled 40 populations across Argentina, Italy, New Zealand, Portugal, South Africa, Spain, and the USA. We developed taxon-specific microsatellite markers using a Next Generation Sequencing approach to analyze the population genetic structure and incidence of hybridization in native and non-native regions. We identified three genetically distinct clusters, which are present in both the native and non-native regions. Based on a set of selected morphological characteristics, we found no clear features to identify taxa morphologically. Our results suggest that the most probable sources of global introductions of *Carpobrotus* species are the Western Cape region of South Africa and the coastline of California. We suggest that management actions targeting *Carpobrotus* invasions globally should focus on preventing additional introductions from the east coast of South Africa, and on searching for prospective biocontrol agents in the Western Cape region of South Africa.

Keywords

Biological invasions, genetic diversity, genetic structure, hybridization, introduction history, invasive alien plant, microsatellite markers, taxonomic uncertainty

Introduction

Coastal habitats such as coastal dunes, sea cliffs, and coastal prairies are exposed to a variety of extreme environmental conditions, including high salinity, low soil moisture, soil nutrient deficiencies, and intense wind and solar irradiance (Maun 2009). These conditions result in a high degree of specialization among species that naturally occur in these habitats (Mayoral et al. 2021). As such, coastal areas often host rare and endemic communities of high conservation value (Acosta et al. 2009). But coastal areas are also among the most endangered habitats (Defeo et al. 2009) and several anthropogenic drivers threaten their conservation, including biological invasions, climate change, habitat degradation, and urbanization (Carboni et al. 2009; Dawson et al. 2017). Invasive plants are considered to be one of the main threats to the conservation of the biodiversity and ecosystem functioning of coastal areas across the world (Millennium Ecosystem Assessment 2005).

The succulent genus *Carpobrotus* N.E.Br. (family Aizoaceae) comprises between 12 and 25 species and lower-rank taxa, most of them native to South Africa (Hartmann 2002). Several of these species are considered to be among the most widespread and damaging invasive plants in coastal areas globally (Campoy et al. 2018). *Carpobrotus* taxa have been introduced to coastal areas accross the world for ornamental purposes and for soil and dune stabilization. For example, they have been present in European gardens

since the late 17th century (Preston and Sell 1988) and, in California, they have been used for soil stabilization since the early 20th century (Albert et al. 1997). *Carpobrotus* species have invaded millions of hectares of coastal areas worldwide, including in Argentina, Australia, California, Chile, New Zealand, and Southern and Western Europe (Campoy et al. 2018), impacting biodiversity and native species community structure and ecosystem functioning in multiple ways. For example, they compete with native plants for space, nutrients and water, reducing their growth, survival, and reproduction (D'Antonio and Mahall 1991; Molinari et al. 2007; Novoa and González 2014). They are also considered ecosystem engineers (Cuddington et al. 2011) since they can cause substantial and irreversible changes to invaded soils (Novoa et al. 2014). In particular, dense patches of invasive *Carpobrotus* produce and accumulate large amounts of litter (Fenollosa et al. 2016), which increases soil water holding capacity and, during its decomposition, decreases soil pH, and increases soil nutrient contents (Novoa et al. 2012, 2014). These changes 'soften' the extreme environmental conditions typical of coastal areas and facilitate the establishment and growth of opportunistic weeds while replacing native coastal vegetation (Novoa et al. 2012, 2013). Invasive *Carpobrotus* also alters the diversity, composition and functioning of soil microbial (Lechuga-Lago et al. 2017; Novoa et al. 2020) and invertebrate communities (Rodríguez et al. 2020; Gutiérrez 2021) and disrupts native pollination (Jakobsson et al. 2008) and herbivory networks (Rodríguez et al. 2019, 2021).

To gain insight into the invasiveness and impact of non-native species, as well as to develop or improve management actions it is important to know the taxonomic identity and the introduction history of the target invasive species (Pyšek et al. 2013). However, the taxonomy and biogeography of *Carpobrotus* spp. have long been a subject of debate (Campoy et al. 2018). Most of the taxa are native to South Africa, but five are native to Australia, and one species (*C. chilensis*) may be native to the Americas. *Carpobrotus* spp. have been described in several floras worldwide (Harvey and Sonder 1861; Blake 1969; Bolus Herbarium Collection 2015; Preston and Sell 1988; Gonçalves 1991; Wisura and Glen 1993), but these lists do not use the same traits to delineate species. The main diagnostic characters used to differentiate species are flower color and shape of the leaf section. However, there are doubts over the validity of these traits for identifying *Carpobrotus* species (Campoy et al. 2018). Thus, the information given in these documents cannot be easily synchronized or compared (Hartmann 2002). Moreover, due to their succulence, *Carpobrotus* spp. are difficult to curate, and therefore are poorly represented in herbarium collections (Walters et al. 2011). In fact, in several cases, the species names are based on lectotypes selected from illustrations, e.g., by Dillenius (1732). As a result, the taxonomy of the genus remains problematic.

Two *Carpobrotus* species are currently considered to be invasive: *C. edulis* (L.) N.E.Br., and *C. acinaciformis* (L.) L.Bolus (Campoy et al. 2018). *Carpobrotus edulis* is the most popular and widely introduced species in the genus. It is native to South Africa and considered one of the worst invasive plants of coastal areas and one of the most thoroughly studied invasive species worldwide (Pyšek et al. 2008; Campoy et al. 2018). It has been reported to hybridize with other *Carpobrotus* species in its native and invasive ranges (hybrids have been documented in the Americas, Australia, Europe, and South

Africa; e.g., Campoy et al. 2018). Hybrids between *C. edulis* and species from other genera (e.g., *Sarcozona*) have also been reported outside South Africa (e.g., Heenan and Sykes 2010). *Carpobrotus acinaciformis* is generally considered to be native to South Africa, although it has also been suggested that it may be a hybrid between *C. edulis* and other South African or Australian congeners (Schierenbeck et al. 2005). *Carpobrotus edulis* and *C. acinaciformis* have a long history of human use in South Africa, and therefore, their natural limits and identities may also be conflated (Malan and Notten 2006).

Carpobrotus chilensis also provides a good example of the taxonomic and biogeographic uncertainties that plague the genus. Some authors consider this species to be native to California and Chile (Brown 1928), while others regard it as native to Argentina and Chile (Hartmann 2002; Zuloaga and Belgrano 2017; US National Plant Germplasm System 2022) and still others suggest it is "probably native to South Africa" (https://ucjeps.berkeley.edu/eflora/eflora_display.php?tid=77164). An extensive review of herbarium and historical records carried out to identify the origin of this species was inconclusive (Bicknell and Mackey 1988). Some authors have even considered it to be a hybrid swarm of five South African species (i.e., *C. deliciosus*, *C. dimidiatus*, *C. edulis*, *C. mellei*, and *C. muirii*) (Bicknell and Mackey 1998). Hence, the origins and taxonomic classification of this taxon are speculative at best. In California, *C. chilensis* has been reported to hybridize with the South African *C. edulis* (Gallagher et al. 1997; Albert et al. 1997; Vilà and D'Antonio 1998) with extensive directional backcrossing and potential loss of pure *C. chilensis* types (Vilà et al. 1998; Schierenbeck et al. 2005). Overall, the genus *Carpobrotus* is often considered to be a complex of species that easily hybridizes and are difficult to distinguish (Traveset et al. 2008). This taxonomic uncertainty is further complicated by the clonal growth typical of the genus, which stabilizes hybrid genotypes (Ellstrand and Schierenbeck 2000).

Here, we aim to shed light on the relatedness and introduction history of invasive *Carpobrotus* spp. around the world. With this overarching aim, we (1) sampled invasive *Carpobrotus* species in coastal areas across many of their presumed native and invaded ranges and (2) developed and used a set of genus-specific microsatellite markers to assess and compare the genetic diversity and structure among these populations. Moreover, aiming to help managers and other stakeholders with the identification of invasive *Carpobrotus* species in the field, we (3) compared the morphological characteristics of the *Carpobrotus* taxa assigned to distinct genetic clusters.

Methods

Study areas and sampling

We sampled a total of 40 *Carpobrotus* populations distributed across their native and invasive ranges (Fig. 1). We considered coastal areas up to 1 km from the sea since these are the typical habitats of invasive *Carpobrotus* taxa (i.e., *C. acinaciformis* and *C. edulis*) (Campoy et al. 2018). According to Hartmann (2002), in South Africa there are six *Carpobrotus* species growing in proximity to the coast: *C. acinaciformis*, *C. muirii* and *C. quadrifidus* found in the Western Cape province; *C. edulis* distributed through

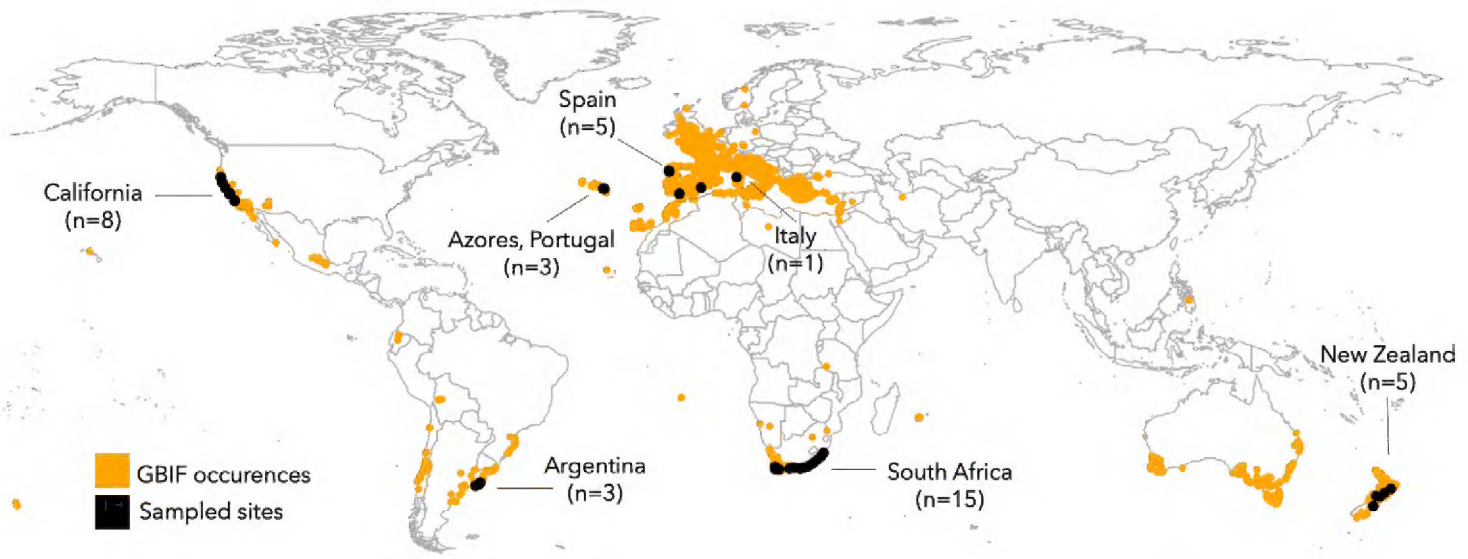

Figure 1. Populations of *Carpobrotus* species sampled in this study (see Table 1 for further details). Orange points indicate occurrence records of invasive *Carpobrotus* species (i.e., *C. acinaciformis* and *C. edulis*) extracted from the GBIF database (gbif.org; downloaded: 31 March 2023; https://doi.org/10.15468/dl.j637g9). Black points indicate the locations where *Carpobrotus* populations were sampled in this study. Numbers in brackets indicate the number of populations sampled per region.

the Northern Cape province, Western Cape and Eastern Cape provinces; *C. deliciosus*, which occurs in the Western Cape, Eastern Cape and KwaZulu-Natal provinces; and *C. dimidiatus*, found in the Eastern Cape and KwaZulu Natal provinces (Smith et al. 1998). Therefore, we selected 15 *Carpobrotus* populations distributed along most of South Africa's coastline (Fig. 1). The selection of the remaining populations was based on the current distribution of invasive *Carpobrotus* species across the world. We extracted information on the current spatial distribution of *C. acinaciformis* and *C. edulis* from the Global Biodiversity Information Facility (GBIF, gbif.org; downloaded: 31 March 2023; https://doi.org/10.15468/ dl.j637g9). We kept records categorized as human observations, literature, living specimens or observations (Fig. 1). Based on these records, we selected 25 additional populations across the observed ranges (Fig. 1, Table 1).

We excluded Chile from our studied area due to issues encountered with exporting plant material from that country. *Carpobrotus* species are also found all along Australia's coastline (Fig. 1). According to Hartmann (2002), four species (i.e., *C. glaucescens*, *C. modestus*, *C. rossii* and *C. virescens*) are native to the country. Moreover, *Carpobrotus* spp. in Australia are hybridizing, both among species within the genus and with species in the genus *Sarcozona* (Campoy et al. 2018). Due to this, and the fact that none of the *Carpobrotus* species native to Australia are recorded as invasive elsewhere in the world, and they are clearly distinct from *C. chilensis* (Bicknell and Mackey 1998), we decided to exclude Australia from our sampling effort.

In each locality (Fig. 1, Table 1), we sampled fresh leaves from about 20 randomly chosen ramets per population (total n = 20 ramets × 40 populations = 800 ramets). The minimum distance between sampled ramets in each population was 5 m. The fresh collected material was shipped to Stellenbosch University, South Africa. Local regulations for sample collection and shipment were followed. Because some samples failed for our molecular analyses, sample sizes for some populations are <20 (Table 1).

Table 1. Locality details and genetic characteristics of populations of *Carpobrotus* species sampled in this study (also see Fig. 1). The region, locality, latitude (Lat) and longitude (Long) in decimal degrees (WGS84), number of samples used for genotyping (N), assigned genetic cluster (Cluster; also see Fig. 4), mean values for the number of alleles per locus (*Na*), observed heterozygosity (H_O), expected heterozygosity (H_E), and inbreeding coefficient (F_{IS}) are provided for each sampled population. For clonal diversity, the number of genotypes (G), number of effective alleles (*Ne*), Nei's (1987) genetic diversity corrected per sample size (H_e) and Nei's uncorrected genetic diversity (Gd) are presented. *diversity-based test not significant, indicating that clonal copies are not necessarily the result of asexual reproduction. The test was significant for all remaining populations (See Methods section for more details). Samples from Cape Point (ZA8) had excessive missing genotype data and were thus removed from the analyses.

ID	Region	Locality	Coordinates (Lat, Long)	N	Cluster	Genetic diversity				Clonal diversity			
						Na	H_O	H_E	F_{IS}	*G*	*Ne*	H_e	*Gd*
NZ1	New Zealand	Whirinaki	-39.829, 176.8914	10	A	2.167	0.483	0.335	-0.440	8	6.250	0.933	0.840
NZ2	New Zealand	Foxton	-40.4557, 175.2168	10	A	2.167	0.533	0.336	-0.490	7	6.250	0.933	0.840
NZ3	New Zealand	Rough Island	-41.2709, 173.1137	14	A	1.667	0.524	0.299	-0.707	4	2.882	0.703	0.653
NZ4	New Zealand	Rarangi	-41.4188, 174.0357	20	A	1.833	0.550	0.336	-0.634	8	4.255	0.805	0.765
NZ5	New Zealand	Lake Ellsmere	-43.8599, 172.3534	20	A	2.000	0.542	0.367	-0.393	11	9.524	0.942	0.895
SE1	Azores	São Vicente	37.8325, -25.6647	30	A	1.500	0.417	0.229	-0.778	1	1.000	0	0
SE2	Spain	Punta de Rons	42.497, -8.8790	16	A	1.500	0.500	0.250	-1.000	1	1.000	0	0
SE3	Spain	A Lanzada	42.4328, -8.875215	24	A	1.500	0.500	0.250	-1.000	1	1.000	0	0
ZA1	South Africa	Rooisand	-34.3490, 19.0909	16	A	2.500	0.469	0.330	-0.344	7	2.415	0.625	0.586
ZA3	South Africa	Vogelgat	-34.4021, 19.3199	16	A	2.500	0.533	0.358	-0.308	7	3.879	0.792	0.742
ZA4	South Africa	Belvidere	-34.0532, 22.9964	13	A	2.000	0.474	0.368	-0.201	8	6.259	0.910	0.840
CA2	California	Celeste	40.8520, -124.1710	23	B	1.333	0.341	0.174	-0.674	1	1.000	0	0
CA3	California	Point Reyes	38.0457, -122.9888	20	B	2.167	0.544	0.431	-0.279	11	8.333	0.926	0.880
CA4	California	For Ord	36.6587, -121.8226	20	B	2.333	0.563	0.427	-0.320	15	10.526	0.953	0.905
CA5	California	Soberanes Point	36.45065, -121.9280	19	B	1.833	0.536	0.358	-0.399	4	2.391	0.614	0.582
CA6	California	Minuteman beach	34.8563, -120.6086	8	B	1.833	0.542	0.296	-0.736	3	2.133	0.607	0.531
CA7	California	Wall beach	34.70521, -120.5995	18	B	2.333	0.576	0.418	-0.341	10	7.364	0.915	0.864
CA8	California	South Base	34.70520, -120.6012	7	B	2.333	0.494	0.422	-0.179	5	3.769	0.857	0.735
SE6	Azores	Ribeira Grande	37.8305, -25.5163	28	B	1.667	0.648	0.333	-0.947	1	1.000	0	0
SE7	Spain	Samil	42.2144, -8.7755	20	B	1.500	0.500	0.250	-1.000	1	1.000	0	0
SE8	Spain	Marina	38.1443, -0.6343	20	B	1.833	0.333	0.212	-0.232	2	1.220	0.189	0.180
ZA5	South Africa	Mdumbi	-31.9443, 29.2100	15	B	1.333	0.333	0.167	-1.000	1	1.000	0	0
ZA10	South Africa	Cape St Francis	-34.1766, 24.8231	8	C	1.667	0.229	0.142	-0.300	4	2.286	0.643	0.562
ZA11	South Africa	Port Elizabeth	-34.0247, 25.6480	19	C	2.167	0.364	0.251	-0.322	7	4.056	0.795*	0.753
ZA12	South Africa	Port Alfred	-33.6093, 26.8900	19	C	1.333	0.225	0.131	-0.606	2	1.870	0.491*	0.465
ZA13	South Africa	Cintsa	-32.8268, 28.1194	19	C	2.000	0.322	0.258	-0.232	3	1.994	0.526	0.499
ZA14	South Africa	Port Edward	-31.0441, 30.2276	18	C	1.500	0.250	0.166	-0.502	4	2.945	0.699	0.660
ZA9	South Africa	Keurboomstrand	-34.0286, 23.3975	20	C	2.000	0.400	0.270	-0.434	8	5.405	0.858	0.815
ARG1	Argentina	Mar Chiquita	-37.7550, -57.4304	22	Admixed	2.000	0.424	0.309	-0.251	4	1.967	0.515	0.492
ARG2	Argentina	San Eduardo del Mar	-38.2355, -57.7548	10	Admixed	2.000	0.412	0.339	-0.254	6	4.167	0.844	0.760
ARG3	Argentina	Quequén	-38.5675, -58.6499	9	Admixed	1.500	0.500	0.250	-1.000	1	1	0	0
CA1	California	Mackerricher	39.4912, -123.7950	16	Admixed	1.500	0.500	0.250	-1.000	3	2.415	0.625	0.586
SE5	Azores	Mosteiros	37.8986, -25.8175	36	Admixed	1.500	0.343	0.184	-0.507	2	1.117	0.108	0.105
SE4	Spain	Cádiz	36.5678, -6.2225	12	Admixed	1.833	0.475	0.315	0.330	4	2.880	0.712	0.653
SE9	Italy	Marina di Sorso	40.8194, 8.4953	21	Admixed	1.833	0.443	0.276	-0.484	6	2.96	0.695	0.662
ZA2	South Africa	Springfontein	-34.4287, 19.4065	10	Admixed	2.333	0.494	0.381	-0.362	10	10	1*	0.900
ZA6	South Africa	Mossel Bay	-34.1715, 22.1226	20	Admixed	2.667	0.507	0.444	-0.171	14	10.526	0.953	0.905
ZA7	South Africa	Melkbosstrand	-33.7065, 18.4482	17	Admixed	2.333	0.331	0.303	-0.048	4	2.513	0.64	0.602
ZA8	South Africa	Cape Point	-34.3530, 18.4888	17	–	–	–	–	–	–	–	–	–
ZA15	South Africa	Durban	-30.1268, 30.8457	18	Admixed	2.500	0.400	0.370	-0.170	8	3.951	0.791	0.747

Microsatellite development and genotyping

Microsatellite sequences were isolated by Ecogenics GmbH (Balgach, Switzerland). Size selected fragments from *Carpobrotus* genomic DNA were enriched for microsatellite repeats by using magnetic streptavidin beads and biotin-labelled CT and GT repeat oligonucleotides. The microsatellite enriched library was analyzed on a Roche 454 Titanium technology (Roche Diagnostics Corporation). This resulted in 89 reads containing microsatellite motifs of at least six microsatellite nucleotide repeat units. Suitable primer design was possible for 32 reads, of which 25 primer pairs were selected and tested for amplification and polymorphism. We extracted DNA from *Carpobrotus* leaf material using a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle 1991). To assess initial amplification success and polymorphism, 25 selected simple sequence repeats (SSRs) loci were first amplified in ten *Carpobrotus* ramets collected in South Africa, using unlabelled primers. Each 10 μL reaction contained 2 μL genomic DNA (100 ng/μl), 1 uL 10× buffer, 200 mM dNTPs, 5 μM of each primer, 1 unit of Taq polymerase (Super-Therm JMR-801, Separations Scientific, Cape Town, South Africa), 0.2 μL bovine serum albumin (BSA, 10 mg/ml) and 3.6 μL of distilled water. The PCR cycling was as follows: 95 °C for 5 min, 30 cycles of 60 s at 94 °C, 60 s at primer-specific annealing temperature, 2 min at 72 °C, and a final elongation of 10 min at 72 °C. To detect polymorphism, the resulting PCR products were purified and run on an Agilent 2100 Bioanalyser analysis LabChip (Agilent Technologies). Out of the 25 loci tested, 19 were monomorphic across all the tested *Carpobrotus* individuals. The forward primers of the remaining six loci were fluorescently labelled with either HEX, 5-FAM, PET, or NED. Primer pairs were combined into two separate multiplex reactions and amplified in all *Carpobrotus* specimens. Each 15 μL multiplex reaction contained 3 μL genomic DNA (20 ng/μL), 1.5 uL primer mix (2 μM), 7.5 μL Qiagen multiplex PCR mix, and 3 μL Q-solution. PCR conditions for all multiplexes consisted of denaturation at 95 °C for 15 min, followed by 30 cycles of 30 s at 94 °C, 90 s at 55 °C, 60 s at 72 °C, and a final elongation of 30 min at 60 °C. Labelled PCR products were sent to the Central Analytical Facility, Stellenbosch University, Stellenbosch, South Africa, for fragment analysis. LIZ500 was used as the internal size standard. GeneMarker software (version 2.6.4; SoftGenetics LLC, Pennsylvania, USA) was used for genotype scoring by using marker panels to call the alleles. All allele scores were checked manually.

Dataset characteristics and genetic diversity

We used the software Micro-Checker (version 2.2; Van Oosterhout et al. 2004) to check our genotype dataset for the presence of scoring errors and null alleles. This software calculates expected homozygote and heterozygote allele size differences by assuming Hardy-Weinberg equilibrium (HWE) conditions, generating the frequency of expected and detected null alleles and applying a Monte Carlo simulation method (Van Oosterhout et al. 2004). Null alleles are identified at a given locus when HWE conditions among genotypes are rejected and if excess homozygous genotypes are evenly distributed among allele size classes. The presence of null alleles can bias calculations of F_{ST} values

and may lead to overestimation of population differentiation (Kim and Sappington 2013). Therefore, for more detailed estimates of null allele frequencies at each locus and population, the expected maximization method as implemented in the software FreeNA (Chapuis and Estoup 2007) was applied. FreeNA was also used to calculate uncorrected and corrected (i.e., excluding null alleles; so-called ENA method as described in Chapuis and Estoup 2007) pairwise F_{ST} values (Weir 1996). For all loci, allele frequency departures from HWE expectations were tested using the packages "adegenet"(version 2.1.1; Jombart 2008) and "pegas" (version 0.11; Paradis 2010) in R (version 3.5.3).

Linkage disequilibrium was evaluated with the "poppr" package (version 2.9.3; Kamvar et al. 2014) by using the index of association of alleles at different loci. For this, measures of correlation ($\overline{r_d}$; Agapow and Burt 2001) were calculated and tested using a permutation approach (n = 1000) and comparing the observed index of association with the expected index value that is independent of sample size. Since linkage disequilibrium can result from clonal reproduction, we calculated the number of clones in each population using the GenoDive program (version 3.06; Meirmans 2020). This was done by calculating the genetic distances between pairs of ramets and using a threshold of genetic similarity. Pairs of ramets falling below this threshold were considered clones. In this case, a threshold of zero (i.e., samples differing in one base pair were considering different clones) was used since it corresponded to the intermediate value between the first peak of frequency of genetic distances (due to possible errors from scoring or somatic mutations) and the second peak of frequencies (Meirmans and van Tienderen 2004). We also considered that errors during genotyping were unlikely, given the low number of alleles per loci we observed. Then, we did a diversity-based test to examine whether duplicated copies were due to asexual or sexual reproduction by randomizing alleles and evaluating the probability that the observed genetic diversity is lower than expected genetic diversity under random mating (the null-hypothesis is that they are similar; Gomez and Carvalho 2000). Lastly, we assessed whether loci had enough power to distinguish among unique genotypes by generating a curve of genotype accumulation using the "poppr" R package. The curve was calculated by doing a random resample (n = 1000) of loci and counting the number of genotypes observed. We also compared the number of unique genotypes present in South Africa to populations from the rest of the world. For these analyses we excluded the population from Cape Point (ZA8) due its very high genetic differentiation from all other populations and incidence of missing data at multiple loci (see Results section). The latter would inflate estimates of clonality.

At the population level, we calculated the number of alleles per locus (*Na*), number of effective alleles (*Ne*), Shannon's index (*I*), and observed and expected heterozygosity (H_O and H_E, respectively). To account for different sample numbers among populations, a rarefaction correction based on the smallest sample size (i.e., population CA8 with seven samples; Table 1) was applied. Further, we calculated the inbreeding coefficient (F_{IS}) for each population. All genetic diversity-related calculations were performed using GenAlex (version 6.5; (Peakall and Smouse 2012). We estimated the number of genotypes (G), number of effective alleles (*Ne*), Nei's (1987) genetic diversity corrected per sample size (H_e) and Nei's uncorrected genetic diversity (Gd) by using GenoDive.

Genetic structure and variation

To investigate the genetic structure among sampled populations, we performed Bayesian assignment tests, as implemented in STRUCTURE (version 2.3.4; Pritchard et al. 2000). We evaluated a range of possible genetic clusters (i.e., *K* values) by using an admixture model with correlated allele frequencies, 100,000 burn-in iterations, 1,000,000 Markov Chain Monte Carlo repetitions and 20 iterations for each value of *K*. To evaluate the optimum number of genetic clusters, we applied the delta *K* method described by Evanno et al. (2005) using the online software STRUCTURE HARVESTER (version 0.6.94; Earl and VonHoldt 2012). Each population was assigned to a specific genetic cluster when the average assignment values of all its individuals was >75% to that cluster. All remaining populations were considered to be admixed. We used the ade4 R package (version 1.7-22; Dray and Dufour 2007) to perform a principal coordinate analysis (PCoA) which was based on the uncorrected genetic distances (Cavalli-Sforza and Edwards 1967) calculated with FreeNA.

Morphology

Aiming to explore whether diagnostic morphological characters could help managers and other stakeholders identify invasive *Carpobrotus* species, we collected data on several morphological characteristics of 10 randomly chosen ramets per sampled population, many of which have been used by previous authors (Albert et al. 1997; Campoy et al. 2018). We selected one flower per ramet and recorded the colour of the petals (Fig. 2A) and filaments of the stamens (Fig. 2B), the shape of the receptacle (i.e., whether the receptacle had a V or U shape; Fig. 2C), the surface of the ovary (i.e., whether the ovary was raised, flat or depressed; Fig. 2D), the position of the calyx globes (i.e., whether the petals are longer, equal or shorter than the sepals; Fig. 2E), and the diameter of the flower (Fig. 2F) and the stamen ring (Fig. 2G). For each ramet, we also selected one leaf located at least two nodes below the apical leaf, and measured its total length, width, and thickness in the centre (Fig. 2H–J, respectively). Finally, we calculated the leaf cross section area as the leaf width, multiplied by the leaf thickness, divided by two.

We then built a regression tree using morphological characteristics as predictors and the genetic cluster to which each population was allocated as the response variable. We excluded those populations with admixed ancestry (Table 1). Regression trees were built using the classification method and pruned, choosing the best complexity parameter. We ran all regression tree analyses using the "rpart" package in R version 4.1.3 (Therneau and Atkinson 2017).

Data resources

The datasets generated during and/or analyzed during the current study are available in https://doi.org/10.5281/zenodo.8123272.

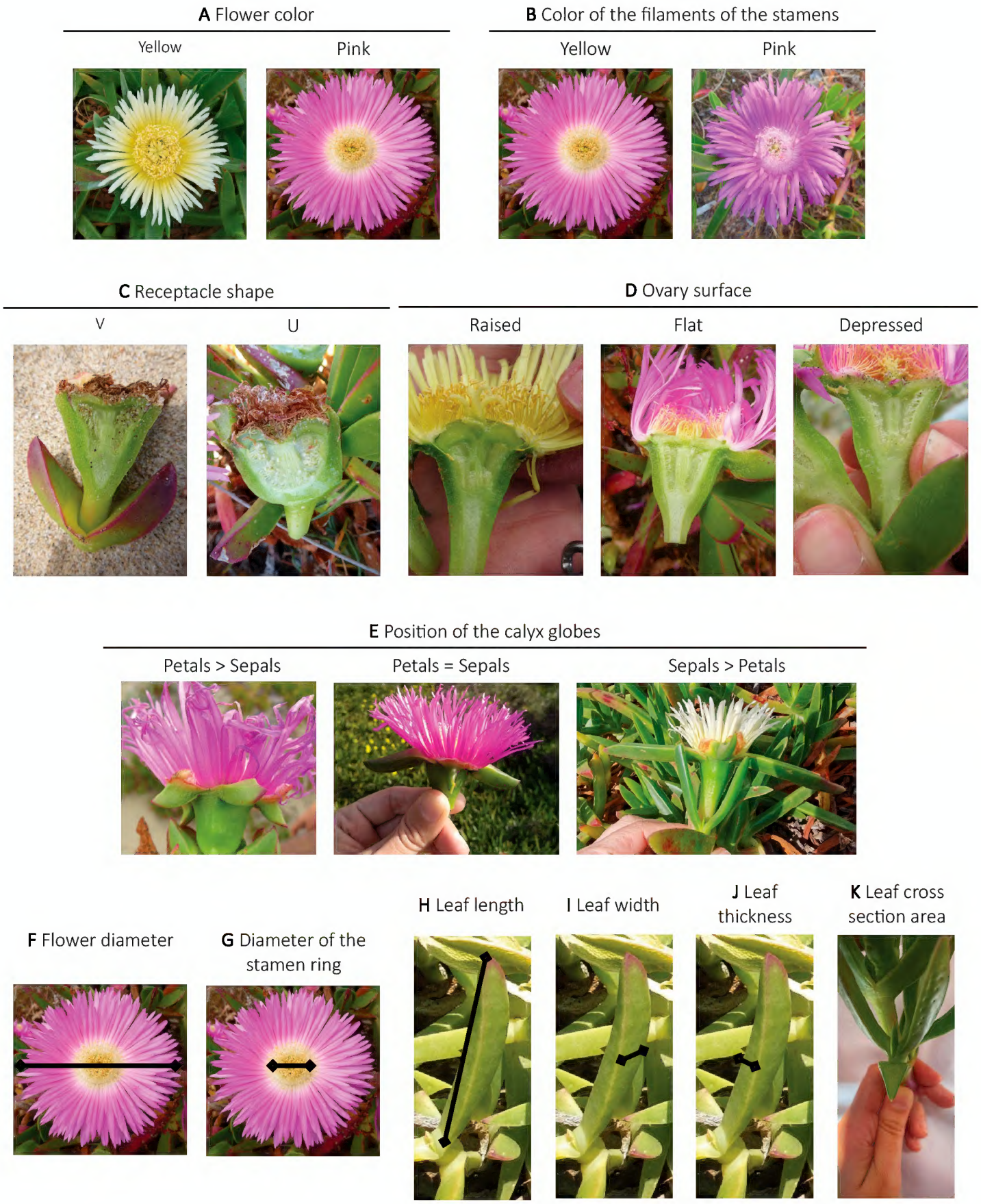

Figure 2. Morphological characteristics of *Carpobrotus* species measured in this study (see text for details).

Results

Dataset characteristics and genetic diversity

We found no evidence of scoring errors due to band stuttering in our genotype dataset. All six loci were polymorphic in the overall dataset and the number of alleles per locus ranged between two and nine.

Samples from Cape Point had a high incidence of missing data and were removed from subsequent analyses. This population likely represents a species that is distantly related to all other species we sampled in our study (average pairwise population F_{ST} = 0.7). For the remaining populations, we found the association index of alleles at different loci to be lower than expected in all populations, indicating the presence of linkage disequilibrium ($\bar{r}_d$ = 0.013; p > 0.001; Suppl. material 2). The GenoDive approach found clones within all populations, with one population from South Africa (Mdumbi) and California (Celestre), two populations from Azores (Ribeira Grande and San Vicente), three populations from Spain (A Lanzada, Punta de Rons and Samil) and one Argentinian population (Quequén) each containing genetically identical ramets (i.e., consisting of a single clone; Table 1). Only one South African population (Springfontein) did not have clones. The diversity-based test confirmed asexual reproduction for all populations except three from South Africa (Port Alfred, Port Elizabeth and Springfontein; Table 1). The genetic accumulation curve showed that the four loci were slightly deficient in distinguishing among genotypes (i.e., 201 of 204 unique multi-locus genotypes were identified after resampling). The maximum number of unique multi-locus genotypes was 204 for the 681 ramets sampled, thus ~70% of sampled ramets were clones. For South Africa, ~53% of sampled ramets were clones.

In all populations, *Na* was low (range 1.4–3.00). Observed heterozygosity was slightly higher (range 0.225–0.648) than H_E (range 0.131–0.444; Fig. 3), and F_{IS} values indicated that all populations have very low or no inbreeding. When comparing populations from the different sampled ranges, levels of H_O, H_E, and F_{IS} were similar.

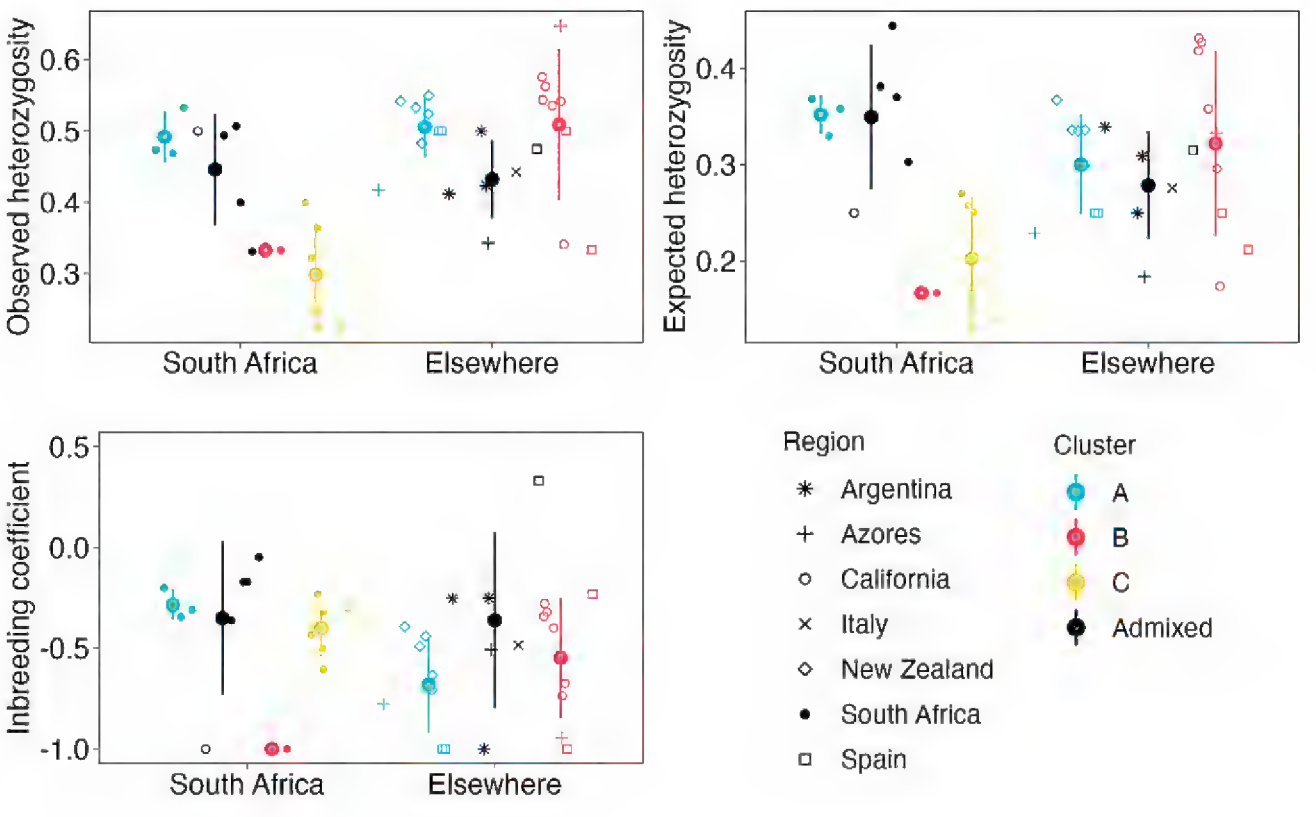

Figure 3. Genetic diversity metrics of native and non-native populations of *Carpobrotus* species. Colours indicate the cluster to which each population has been assigned (See Table 1 and Fig. 4 for further details). Although admixed populations are not necessarily genetically similar, they were combined for visualization purposes.

No differences in the level of these indices were found between population ranges of each cluster, except that H_O and H_E were lower in populations of cluster C (which were all South African populations) than in other populations (see below for genetic structure results, Table 1 and Fig. 4). For clonal diversity, *Ne* was low for most populations while *He* and *Gd* were relatively high.

Genetic structure

Population pairwise F_{ST} estimates (excluding population ZA8 from Cape Point) ranged from low (F_{ST} = 0.015; between populations ZA1 and ZA2) to high (F_{ST} = 0.6; between populations ZA5 and ZA10) (Suppl. material 1). The results of the STRUCTURE analysis, including 40 sampled populations identified K = 3 as the optimal number of genetic clusters (Fig. 4). Based on the criteria outlined in the Methods we identified 11 populations as being admixed, with the remaining populations having high overall assignment values to one of the three identified genetic clusters only. Although we found all three genetic clusters in South Africa, two of them were more dominant: cluster A along the west coast and cluster C along the east coast. In South Africa, only one population (Mdumbi) was clearly associated with cluster B (Fig. 5).

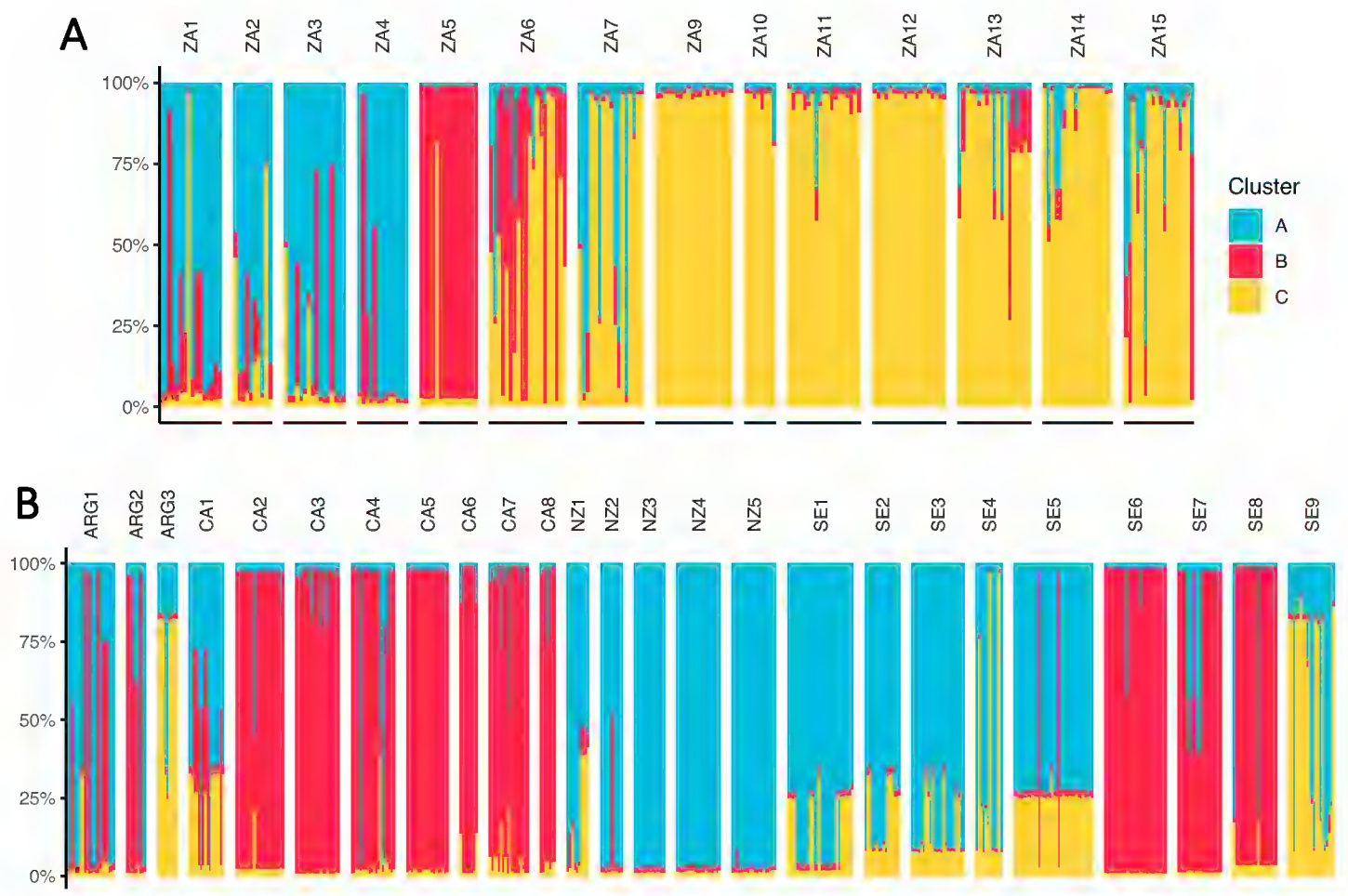

Figure 4. Bar plots showing the genetic structure of the **A** native South African and **B** non-South African populations of *Carpobrotus* species included in this study. Note that both plots represent the same analysis and were split into two panes for better visualization. The delta *K* method following Evanno et al. (2005) revealed K = 3 as the optimal number of genetic clusters. Abbreviations above the bar plots indicate the ID of the populations (see Table 1 for more details).

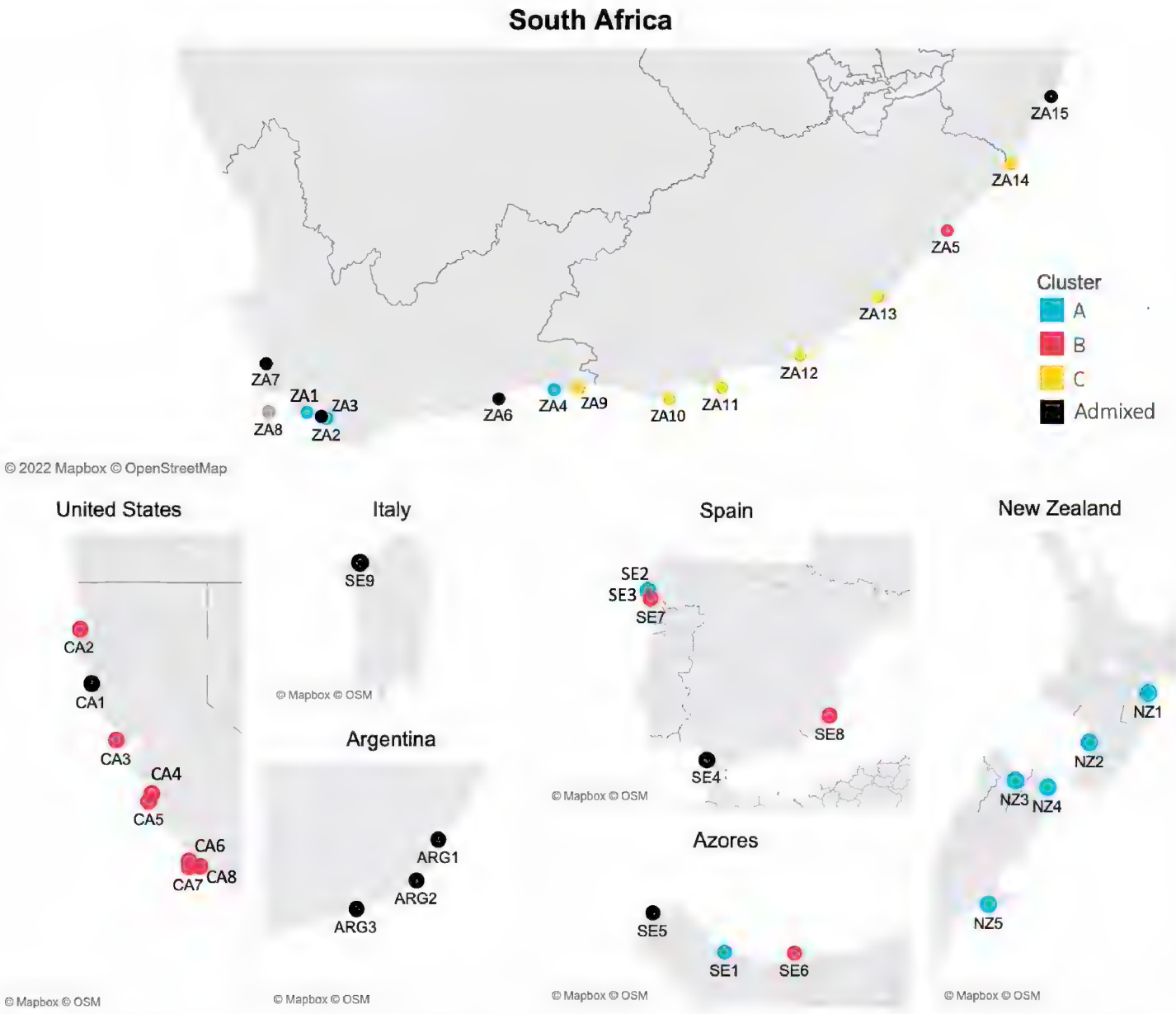

Figure 5. Geographical distribution of the populations of *Carpobrotus* species sampled in this study (see Table 1 for further details). Points roughly indicate the location of the populations. The colour of the points indicates the main cluster each population was assigned to (See Table 1 and Fig. 4 for further details). Cape Town population (ZA8) is indicated in grey since it had excessive missing data and, therefore, was removed from the analyses.

All five populations from New Zealand, two from Spain and one from the Azores (Portugal) appeared to be more closely related to west coast populations from South Africa (i.e., genetic cluster A). Six of the seven populations sampled in California (USA), together with one population from South Africa, two from Spain and one from the Azores (Portugal), formed a separate cluster (cluster B). A similar pattern was also observed in the PCoA, although one of the Spanish populations (i.e., SE8) was assigned to cluster B by the STRUCTURE analysis, and showed no clear association with any of the studied populations (Fig. 6). Moreover, the PCoA results showed one of the sampled Argentinian populations (i.e., ARG2) to be similar to those assigned to cluster B by the STRUCTURE analysis, while one population from Argentina (i.e., ARG3), one from California (i.e., CA1) three from Europe (i.e., SE4, SE5 and SE9) and one from South Africa (ZA2), previously classified as admixed, clustered with those assigned to cluster A.

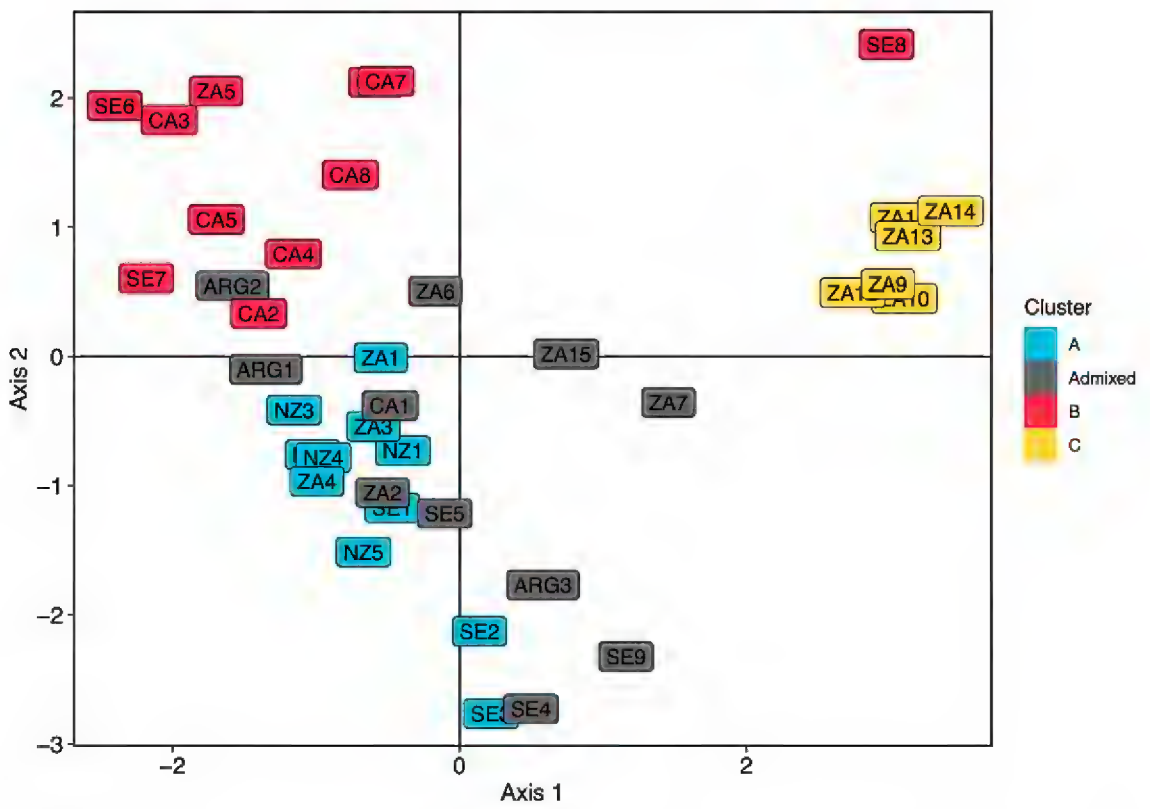

Figure 6. Principal coordinates analysis for the populations of *Carpobrotus* species included in this study. The analysis was based on genetic distances (following Cavalli-Sforza and Edwards 1967) between populations. Populations are indicated with different colours according to the main cluster they have been assigned to or levels of admixture (see Table 1 and Fig. 4).

Morphology

We found no clear link between morphological characteristics and the identified genetic clusters of *Carpobrotus* plants sampled in our study (Figs 7, 8). However, individuals belonging to cluster A generally present yellow stamens (62.8% of the sampled individuals), their sepals are longer or the same length than the petals (90.9%), and the diameter of their stamen ring is longer or equal to 2.1 cm (81%). Individuals assigned to cluster B always present yellow stamens, generally have a flat or raised ovary (95.3%), and their leaves are normally shorter than 10 cm (98%). The individuals assigned to cluster C have pink flowers, and generally present pink stamens (72%), generally have depressed ovaries (68%), their petals are generally longer or equal in length to their sepals (86%), and their stamen ring is generally smaller than 2.1 cm (78%). However, there were many exceptions to these patterns (Figs 7, 8).

Discussion

Our results confirm the complex identification, biosystematics and biogeography of the invasive *Carpobrotus* spp. The west coast of South Africa, and possibly California, were identified as the most likely sources of invasive populations worldwide.

The Bayesian assignment analysis grouped the sampled populations into three genetic clusters (clusters A, B and C; Fig. 3). In South Africa, the native distribution area of most *Carpobrotus* spp. (Germishuizen and Meyer 2003), most sampled individuals

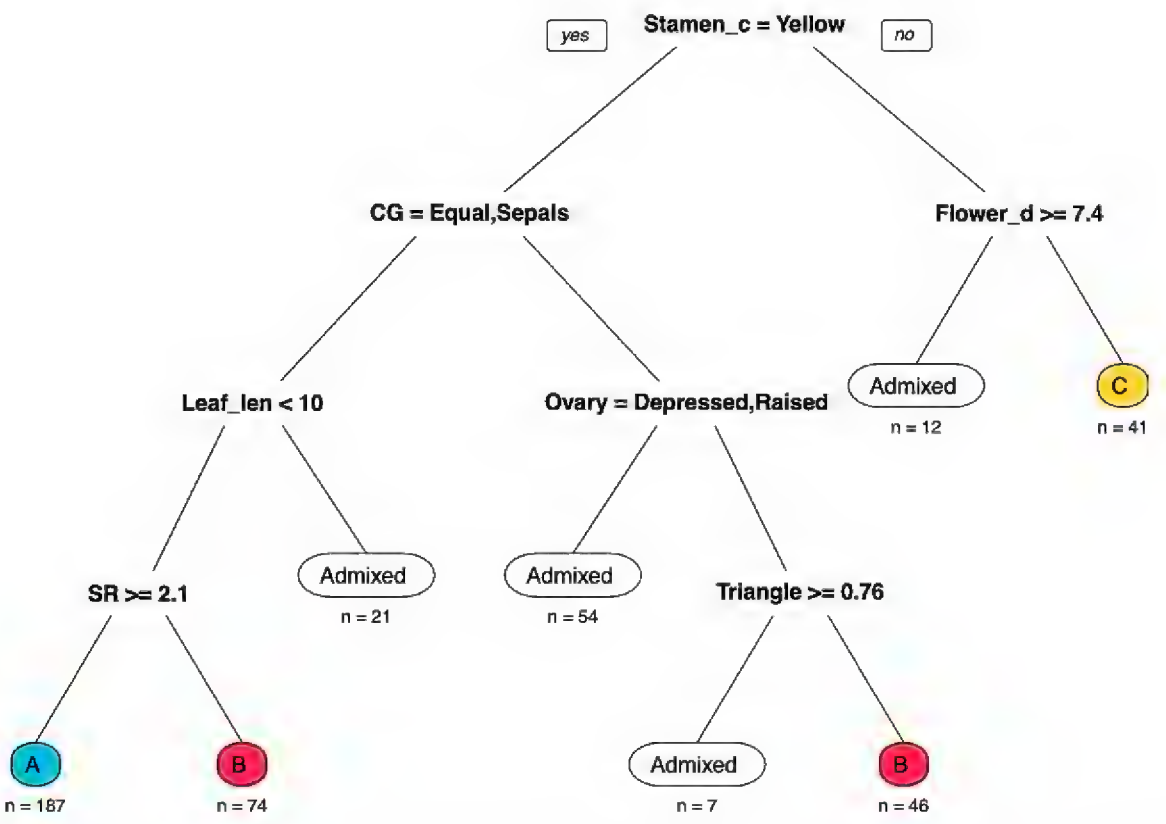

Figure 7. Classification tree analysis of the *Carpobrotus* genetic clusters based on morphological characteristics. The most significant characteristic is indicated at each node, with the corresponding values relating to branches on the left. Morphological differences between genetic clusters could be best explained by the color of the filaments of the stamens (Stamen_c), the flower diameter (Flower_d), the position of the calyx globes (CG), the ovary surface (Ovary), the leaf cross section area (Triangle), the leaf length (Leaf_len) and the diameter of the stamen ring (SR). Leaf_len, Flower_d and SR are indicated in cm. Colours of circles at the end of branches correspond to the genetic clusters. *n* = number of individuals assigned to each cluster. See Fig. 2 for details on the morphological characteristics included in the analysis.

were assigned to clusters A (in the Western Cape province) and C (in the Eastern Cape and Kwazulu-Natal provinces). Four *Carpobrotus* species (including their described lower taxa) are considered native to the Western Cape province: *C. acinaciformis*, *C. edulis*, *C. muirii*, and *C. quadrifidus* (Smith et al. 1998). Individuals assigned to cluster A could therefore correspond to one or several of these species, or to hybrids between them. On the other hand, two species occur naturally in the Eastern Cape and KwaZulu-Natal provinces: *C. deliciosus* and *C. dimidiatus* (Smith et al. 1998). Therefore, cluster C likely corresponds to individuals of one or both of these two species or hybrids between them.

Only one South African population, consisting of a single genotype, was assigned to cluster B (shared by some populations from southern Europe and California; Table 1). This population is located in Mdumbi, a remote area that attracts tourists from all over the world due to the presence of various ecotourism establishments and surfing lodges (Hitchcock 2014). Cluster B was predominantly found in California, although two populations from Spain and one in the Azores were also from this cluster. These results suggest that populations assigned to cluster B might have originated from South Africa decades ago, introduced to California directly from South Africa or secondarily via Spain (i.e., a country with an extensive history of trade with the California coast; Engstrand 1997), and hybridized extensively (Vilà et al. 1998; Schierenbeck et al.

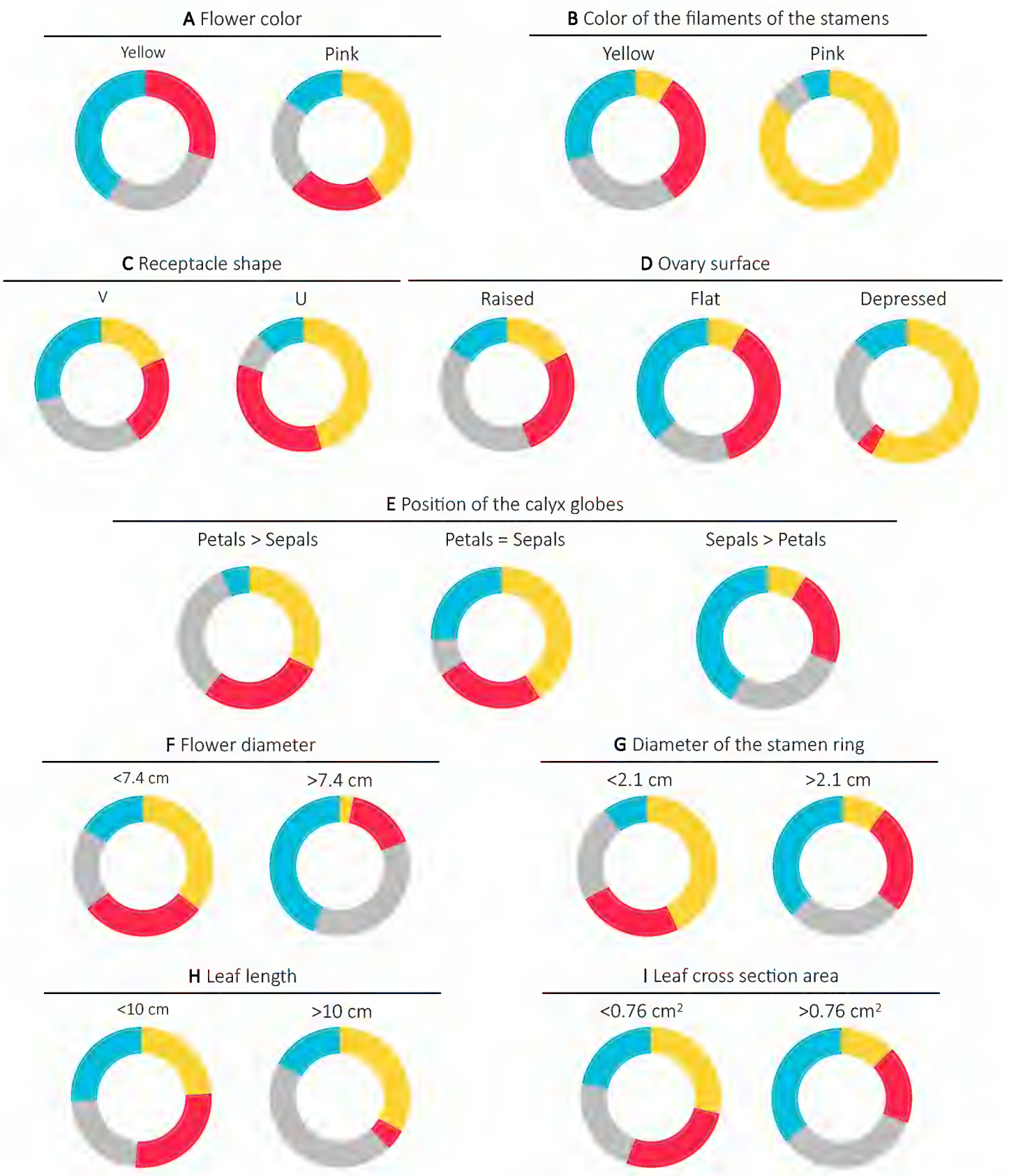

Figure 8. Donut plots representing the proportion of *Carpobrotus* individuals belonging to different genetic clusters, or admixtures between them, and morphological characteristics (see Fig. 2 for details). Blue = cluster A; red = cluster B; yellow = cluster C; grey = admixed.

2005) with *C. chilensis*, a species of unknown origin that mainly occurs in the Pacific coasts of the American continent (Campoy et al. 2018). The abrupt appearance of *C. chilensis* pollen within a 900-year-old record for the central California coast in the early 1800s suggests introduction with early Spanish settlement or visitation including extensive migration of people from Portugal from the Azores to coastal California in the 1800s (Williams 1982; Bicknell and Mackey 1988). Individuals representing cluster B

may then have been introduced to the coast of Spain and/or the Mdumbi region after hybridization had occurred with the later introduced *C. edulis*. It is also possible that the *C. chilensis* plants in California arose from an early introduction from an unknown source where the species no longer exists and then pure *C. chilensis* in California has largely disappeared through hybridization.

Outside South Africa and California, all sampled populations were assigned to either cluster A or B, or were identified as admixed. These findings suggest that the Western Cape province of South Africa and coastal California may have served as the sources for many introduced *Carpobrotus* populations in the rest of the world. This is not surprising, given that *Carpobrotus* species have been widely introduced as ornamental plants (Campoy et al. 2018), and both regions have been prominent hubs of the ornamental horticulture industry for centuries (University of California 1999). More specifically, all individuals sampled in New Zealand were assigned to cluster A, suggesting a South African origin of *Carpobrotus* invasions in this country. In Spain and Azores, most populations were assigned to clusters A and B, suggesting multiple introductions from South Africa and the Americas.

The Italian and Argentinian populations included in our analyses were not clearly assigned to particular genetic clusters, suggesting that genetically distinct groups or species of *Carpobrotus* were introduced to these areas from different sources, leading to extensive admixture (Suehs et al. 2004). Accordingly, hybridization has been repeatedly suggested to play an important role in the invasiveness of *Carpobrotus* species (Campoy et al. 2018), with hybrids having higher survival and faster growth rates than parental taxa (Vilà and D'Antonio 1998). Moreover, our results show that hybridization is also common in South Africa, the native range of most species in the genus. The implications of hybridization for the invasion of *Carpobrotus* are poorly understood and deserve further research attention.

Overall, our results indicate that there have been multiple introductions of *Carpobrotus* species from different sources globally. Typically, multiple introductions increase the genetic diversity and probability of success of invasive species (Genton et al. 2005; Walls 2010). However, we found extremely low levels of genetic diversity in all studied populations. The reason for this can probably be attributed to the capacity for self-fertilization (Vilà et al. 1998) and the clonal nature of *Carpobrotus* species, which facilitates vegetative reproduction without genetic recombination (Campoy et al. 2018) which typically results in low genetic diversity (e.g., Hollingsworth and Bailey 2000). Accordingly, we observed low inbreeding and high clonality levels in all sampled populations. Moreover, clonality has been suggested to allow *Carpobrotus* species, and alien plants in general, to effectively establish and colonize new areas (Roiloa et al. 2010). These observations also explain the high number of monomorphic loci we identified during genetic marker development and testing.

Accurate identification of invasive *Carpobrotus* species or hybrid combinations could improve risk assessment and guide early detection and rapid response management actions (Guisan and Thuiller 2005). For example, in California, some managers do not want to remove what seems to be *C. chilensis* because they do not know whether

or not it is native and it appears to coexist with native species and can be helpful in dune stabilization (D'Antonio, personal observation). Also, "taxonomic identity" should be specified in any risk assessment/analysis scheme (e.g., IPPC, ISPM 2, Framework for pest risk analysis) and local management plans for the removal of species. Similarly, Species Distribution Models (SDMs) used to guide early detection and rapid response actions typically use distributional data of the target species, coupled with characteristics of the current and potentially suitable areas (e.g., climate, land-use type) (Guisan and Thuiller 2005). Using Ecological Niche Models, Thuiller et al. (2005) identified areas of high suitability for invasive *Carpobrotus* species in Australia, central east Africa, Chile, Europe and the USA. However, it is conceivable that the geographic extent of such predictions depends on the occurrence records of the *Carpobrotus* species and/or their hybrids used to calibrate these models. Accurate identification and knowledge of the introduction history of invasive *Carpobrotus* spp. are also critical for reducing the negative impacts of their current invasions. The most common methods used to control *Carpobrotus* invasions include mechanical and chemical methods (Ruffino et al. 2015). However, these methods require large amounts of funding and capacity, follow-ups and restoration efforts, and have not been successful at reducing *Carpobrotus* invasions at large geographic scales. The integration of biological control into the management of invasive *Carpobrotus* species could reduce management costs significantly and increase management success (Campoy et al. 2018). For example, the South African soft scale *Pulvinariella mesembryanthemi* (Vallot, 1829) is a specialist herbivore of *Carpobrotus* spp. that was accidentally introduced into California, causing considerable damage to invasive populations of *C. edulis* (Washburn and Frankie 1985) where it also became a pest of the presumed native *C. chilensis* (Schmalzer and Hinkel 1987). Subsequently, predators and parasites were released from South Africa to control the scale (Tassan et al. 1982). *Pulvinariella mesembryanthemi* is still a promising potential biological control agent outside of California (Vieites-Blanco et al. 2019; Núñez-González et al. 2021). But the effectiveness of *P. mesembryanthemi* is likely to depend on the taxonomic identity and source region of the target species (Pyšek et al. 2013; Le Roux 2021).

However, identifying invasive *Carpobrotus* species is challenging. Several diagnostic morphological characters have been proposed to differentiate between species, with petal colour being the most popular one (Preston and Sell 1988; Wisura and Glen 1993), but doubts have been expressed on the validity of all proposed characters as taxonomic markers (Campoy et al. 2018). Our results show no clear pattern regarding the association of morphological traits with the three genetic clusters we identified. We only collected morphological data from 10 individuals per population in the field, and each population was located in a different coastal habitat (e.g., disturbed areas or dunes). The different conditions to which *Carpobrotus* individuals were exposed in the field might have added a large variation to our morphological results. Additionally, within some populations, variation between individuals was high, potentially swamping differences across populations. Moreover the widespread occurrence of hybrid populations makes identification using morphological data even more difficult (Suehs et al. 2004).

Despite the challenges related to the morphological identification of invasive *Carpobrotus* species using morphological characters, our results have important implications for the development of management programmes. First, no introductions of individuals from cluster C have been detected in any of the sampled sites. However, the rate of introduction of alien species is rapidly increasing (Seebens et al. 2021). This, coupled with the widespread use of *Carpobrotus* species as ornamental plants, enhances the chances of individuals from cluster C to be introduced and the potential for genetic exchange between populations from all three clusters, which could increase the invasion success of *Carpobrotus*. Hence, management strategies should aim at preventing the introduction of additional *Carpobrotus* genotypes, especially from the Eastern South African coast. Second, we revealed that the most probable sources of *Carpobrotus* introductions and invasions globally are the Western Cape province in South Africa and California. Since most effective biocontrol agents are generally those that have co-evolved with the invasive species (Müller-Schärer et al. 2004), the search for biocontrol agents to manage *Carpobrotus* invasions should be focused in these areas. A challenge for this in California is the fact that pure *C. chilensis* is rare due to the extensive hybridization, and no specialist insects have been observed on it other than the rare occurrence of the introduced scale insects *Pulvinariella mesembryanthemi* and *Pulvinaria delottoi* (Schmalzer and Hinkel 1987). Moreover, there is no clear evidence that *C. chilensis* is native to California, and future studies should extend sampling efforts to other areas such as the coasts of Chile and Australia. Additionally, it should be carefully explored whether biocontrol agents from the Western Cape province in South Africa and California are effective at managing admixture (or hybrid) populations or *Carpobrotus* invasions in general.

Our work highlights exciting opportunities for future research on *Carpobrotus* invasions. For example, high-resolution population genomic analyses (e.g., single nucleotide polymorphism genotyping or whole genome sequencing), coupled with common garden experiments, would provide valuable insights into the diversity and evolutionary dynamics of the genus, the invasiveness of its representatives and their interactions with insects with the potential to be used for biological control. For instance, a highly flexible breeding system that allows extensive hybridization (i.e., outcrossing) and high levels of clonal reproduction (via vegetative structures) suggest the stabilization of highly successful hybrid genotypes is likely to occur. Determining whether certain hybrid combinations and/or clones are more prevalent in native or invasive ranges should be included in future research to inform future management of the group.

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Supplementary material 1

Pairwise estimates of genetic differentiation based on microsatellite data (FST)

Authors: Ana Novoa, Heidi Hirsch, María L. Castillo, Susan Canavan, Luís González, David M. Richardson, Petr Pyšek, Jonatan Rodríguez, Lurdes Borges Silva, Giuseppe Brundu, Carla M. D'Antonio, Jorge L. Gutiérrez, Megan Mathese, Sam Levin, Luís Silva, Johannes J. Le Roux

Data type: xlsx

Explanation note: Pairwise estimates of genetic differentiation based on microsatellite data (FST) for all populations included in this study. Note that 'population ID' refers to the same IDs provided in Table 1 in the main manuscript.

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Link: https://doi.org/10.3897/neobiota.89.109164.suppl1

Supplementary material 2

Testing for linked disequilibrium on 681 samples of *Carpobrotus* species

Authors: Ana Novoa, Heidi Hirsch, María L. Castillo, Susan Canavan, Luís González, David M. Richardson, Petr Pyšek, Jonatan Rodríguez, Lurdes Borges Silva, Giuseppe Brundu, Carla M. D'Antonio, Jorge L. Gutiérrez, Megan Mathese, Sam Levin, Luís Silva, Johannes J. Le Roux

Data type: pdf

Explanation note: The blue line ($\overline{r}_d$) indicates the expected index of association of alleles at different loci that is independent of sample size. A distribution below $\overline{r}_d$ indicates linkage disequilibrium and evidence for clonal reproduction.

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